Long Endogenous dsRNAs Can Induce Complete Gene Silencing in Mammalian Cells and Primary Cultures

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ABSTRACT

Recently, double-stranded RNA (dsRNA)-mediated RNA interference (RNAi) has rapidly developed to a powerful instrument for specific silencing of gene expression in several organisms, including *Caenorhabditis elegans, Drosophila melanogaster*, and plants. The finding that synthetic small interfering RNAs (siRNAs) of 21 nt as well as stable, endogenously expressed, large dsRNA are suited to specifically induce gene silencing in mammalian cells offered the possibility of expanding this technique to mammalian systems. In this work, we engineered stably transfected human cells that express large dsRNAs mediating specific posttranscriptional silencing of genes. We used this technique to specifically silence genes coding for glucosylceramide synthase (GCS), the sphingolipid activator protein precursor (SAP), and glucocerebrosidase (GBA), all implicated in glycosphingolipid metabolism. From a 1600-bp inverted repeat DNA template, a dsRNA of 800 bp is expressed and predicted to mediate the specific suppression of the corresponding gene by RNAi. Remarkably, we were able to use this method to achieve complete inhibition of those genes we targeted in different cultured human cell lists. These findings testify to the generality of RNAi application in suppressing gene expression in mammalian cells.

INTRODUCTION

RECENTLY, RNA INTERFERENCE (RNAi) has become a very potent method to posttranscriptionally silence gene expression in order to investigate protein function. It is induced by the introduction of double-stranded RNA (dsRNA) and its processing to 21–23-nt small interfering RNAs (siRNAs), which cause the degradation of homologous mRNA. However, it is still difficult to apply RNAi to mammalian organisms. dsRNA is able to target homologous endogenous mRNA for sequence-specific cleavage, blocking its translation into the corresponding protein. It is processed by an RNase III homologous enzyme called Dicer (Bernstein et al., 2001) into small siRNAs of 21–23 nt that are the active triggers of RNAi (Elbashir et al., 2001). After their formation, these siRNAs are recruited by a multiprotein complex, with which they form a ribonucleotide-protein complex (RNP). Within this RNP, the siRNA are unwound by ATP-depending helicase activity, leading to the formation of the dsRNA-induced silencing complex (RISC) (Nykanen et al., 2001) that targets the endogenous mRNA for sequence-specific cleavage.

Because dsRNA can induce the sequence-specific cleavage of homologous mRNA, this method has become a powerful instrument for reverse genetic studies in several invertebrate species, including *Caenorhabditis elegans* and *Drosophila*, and in plants. In these organisms, efficient specific silencing of gene expression has been obtained by the use of large exogenous dsRNA (Fire et al., 1998; Misquitta and Paterson, 1999; Tuschl et al., 1999). It was shown, however, that application of large dsRNA to mammalian cells causes apoptotic cell death. This is because dsRNA longer than 30 nt induces the activation of protein kinase R (PKR), which phosphorylates and inactivates the translation factor eukaryotic initiation factor 2 (eIF2α), leading to a generalized shutdown of protein biosynthesis (Lee et al., 1994, 1997). The first

successful attempts to apply RNAi in cultured mammalian cells came from Tuschl's group (Elbashir et al., 2001). To overcome the problem of the large dsRNA-dependent nonspecific interferon (IFN) response, they used synthetic siRNAs of 21 nt. However, the use of these siRNAs is transient, which severely restricts its applications. To overcome this limitation, several groups generated mammalian expression vectors that direct the synthesis of endogenous dsRNA or siRNA-like transcripts (Brummelkamp and Agami, 2002; Yu et al., 2002) and show similar potency to trigger RNAi in mammalian cells. These siRNA-expressing plasmids were designed for either *in vitro* transcription of siRNA hairpins using an upstream T7-polymerase recognition sequence, which were eventually transfected into the cells (Yu et al., 2002), or for stable expression of the siRNA hairpin transgenes driven by such strong promoters as cytomegalovirus (CMV) or elongation factor (EF1α) or a strong RNA polymerase III (pol III) promoter, such as U6 and H1 (Brummelkamp and Agami, 2002; Lee et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002a; Paul et al., 2002; Sui et al., 2002).

These vector systems mediate permanent suppression of specific gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time. Moreover, vector-mediated RNAi may allow therapeutic applications by permanent suppression of pathogenic proteins. However, in none of these attempts was a 100% knockdown of the activity of the targeted gene achieved. Tuschl's group realized a knockdown of only 80%–90% by using their synthetic siRNAs. Although short dsRNAs do not mediate RNAi efficiently (Parrish et al., 2000; Tuschl et al., 1999; Yang et al., 2000), regions of homology between the dsRNA and the target gene as short as 23 nt can mediate posttranscriptional gene silencing in tobacco, when the homology is contained within a longer dsRNA. The fact that purified siRNAs cleaved from long dsRNA (Nykanen et al., 2001) can efficiently mediate RNAi *in vitro* suggests that long dsRNAs are more effective because they are more efficiently processed into siRNAs. This can result from a highly cooperative binding.

Notably, Hannon's group engineered transgenic mice cells expressing a dsRNA of 500 bp with which they were able to specifically target different genes for posttranscriptional gene silencing (Paddison et al., 2002b; Paddison and Hannon, 2002).

In this work, we used a DNA plasmid-based approach to achieve specific stable posttranscriptional silencing (RNAi) of the glucosylceramide synthase (GCS), the β-glucosidase (GBA), and the sphingolipid activator precursor (pSap) genes that encode proteins involved in glycosphingolipid metabolism (Diallo et al., 2003; Kolter, 1999; Schuette et al., 2001). To assess the ability of en-

dogenously expressed dsRNA of several 100 bp to trigger RNAi in mammalian cells, we generated different stably transfected human cell lines stably expressing an 800-bp dsRNA homologous to one of the endogenous mRNA. These dsRNAs are predicted to target the corresponding mRNA for degradation. Remarkably, in all three cases, we were able to obtain cell clones that show complete inhibition of gene expression, indicating that this procedure considerably facilitates the application of RNAi technology in cultured mammalian cell lines and primary cells.

MATERIALS AND METHODS

Cell culture

Mammalian cell lines (HeLa, mouse melanoma cells [MEB4], and Gaucher type II primary fibroblasts) were cultured in DMEM (Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany) and penicillin/streptomycin (1%) at 37°C and 5% CO_2. Primary fibroblasts were obtained from a skin biopsy of a Gaucher patient. The skin biopsy was cut in small slices under sterile condition in Hank's balanced salt solution (HBSS) and treated with trypsin (0.25% tryspin/2% EDTA in phosphate-buffered saline [PBS]). The slices were cultured in a 25-cm² cell culture flask precoated with 1 ml FBS using DMEM supplemented with 10% FBS and penicillin/streptomycin (1%) at 37°C and 5% CO_2. After outgrowth of fibroblasts, the culture was again trypsinized and transferred into a new flask to accelerate the growth of fibroblasts.

Total RNA preparation

Cells were grown to confluency in 6-well plates. After removal of the medium cells were washed with 1× PBS, and total RNA was prepared using the RNeasy RNA-extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's directions.

Cloning and transfection of pGCShp-cDNA3.1, pSAPhp-cDNA3.1, and pGBAhp-cDNA3.1

Using total RNA, we first generated two 800-bp long DNA fragments corresponding to the first 800 bp of the respective cDNAs by RT-PCR. GCS: primers for sense orientation: forward, *BamHI* 5'-GAGT<u>GGATCC</u>AGA-TGGCGCTGCTGGACCTGGCCTTGGAG-3'; reverse, *EcoRI* 5'-GAGT<u>GAATTC</u>TTATACATCTAGGATTT-CCTCTGCTG-3'; for antisense orientation, forward, *XhoI* 5'-GAGTTA<u>CTCGAG</u>ATGCGCCTGCTGGAC-CTGGCCTTGGAG-3'; reverse, *EcoRI* 5'-GAGT<u>GAA-TTC</u>TTATACATCTAGGATTTCCTCTGCTG-3'.

SAP: primers for sense and antisense orientation: forward, *BamHI* 5′-CGAC<u>GGATCC</u>AGATGTACGCC-CTCTTCCTCCTGGCCAGC-3′; reverse; *EcoRI* 5′-GT-AC<u>GAATTC</u>AGCGCACAGATCTCCTTGGGTTGC-ATG-3′. GBA: primers for sense and antisense orientation: forward, *EcoRI* 5′-GGA<u>GAATTC</u>ATGGAGTTTTCA-AGTCCTTCCAGAGAG-3′; reverse, *BamHI* 5′-CGA<u>G-GATCC</u>CTGCCCAGAACTGTAACTTGTGCT-CAGC-3′. The RT-PCR was performed using the Titan One Tube RT-PCR system (Roche, Mannheim, Germany) according to their instructions. The following PCR conditions (PCR cycler, MJ Research, Newton, MA) were used: 56°C for 35 minutes and 94°C for 2 minutes; 10 cycles of the following steps: 94°C for 20 seconds, 58°C for 45 seconds, 68°C for 2 minutes; 25 cycles of the following steps: 94°C for 20 seconds, 62°C for 45 seconds, 68°C for 2 minutes; end fillup 68°C for 7 minutes. To combine sense and antisense oriented strands for GCS, both fragments were inserted into the *BamHI* and *XhoI* sites of the eukaryotic expression plasmid pcDNA3.1 (Invitrogen), generating the plasmid pGCShp-cDNA3.1, into the *BamHI* site for pSAPhp-cDNA3.1, and into the *EcoRI* site for pGBAhp-cDNA3.1. An *EcoRI* site (*BamHI* site for GBA) as an inversion point restriction site between the sense and antisense orientation supported directional cloning and served as a loop.

Mammalian cell lines were grown to 50%–60% confluency. Before transfection, they were trypsinized, and cells were transfected with the pGCShp-cDNA3.1, pSAPhp-cDNA3.1, and pGBAhp-cDNA3.1 transgenes in suspension using FuGENE6 (Roche) according to the manufacturer's instructions. Cells were plated into 6-well plates using DMEM supplemented with 10% FBS and penicillin/streptomycin (1%) and incubated at 37°C and 5% CO_2. Cell clones that were positive for the pGCShp-cDNA3.1, pSAPhp-cDNA3.1, and pGBAhp-cDNA3.1 DNA were selected using a gradient (300–1200 μg/ml) of the neomycin derivative G418 concentration for 30–40 days.

RT-PCR

RT-PCR analysis of residual mRNA in the RNAi cells was performed as described using the following primers. GCS: forward, 5′-GAGTGGATCCAGATGGCGCTGC-TGGACCTGGCCTTGGAG-3′; reverse, GAGTGA AT-TCTTATACATCTAGGATTTCCTCTGCTG-3′; pSAP: forward, 5′-CGACGGATCCAGATGTACGCCCTCTT-CCTC CTGGCCAGC-3′; reverse 5′-GAACGAATTCC-TAGTTCCACACATGCAATGCTCGACAG-3′; GBA: forward, 5′-GGAGAATTCATGGAGTTTTCAAGTCC-TTCCAGAGAG-3′; reverse 5′-GTGTAACTCGAGTCA-CTGGCGATGCCAACGGTAGGTGTG-3′; 2′5′-oligoadenylate synthase: forward, 5′-AGGTGGTAAAGGG-

TGGCTCC-3′; reverse, 5′-GACTAATTCCAAGACC-GTCC-3′.

Western blot analysis

Wild-type and pGCShp-cDNA3.1 cells were grown to confluency of about 50%–60% on 6-well plates. After removal of the medium, the cells were washed with PBS and lysed with 400 μl denaturing SDS-PAGE buffer. Homogenized samples were heated for 5 minutes at 95°C and separated in a 12.5% SDS-PAGE. Following SDS-PAGE, the gel was equilibrated for 20 minutes in transfer buffer (10 mM 3[cyclohexyl-amino]-1-propanesulfonic cid [CAPS], pH 11, and 10% methanol) and blotted onto a PVDF membrane. Immunodetection was performed using a rabbit anti-GCS antibody (1:500) (kindly provided by R. Pagano) as a primary antibody and a horseradish peroxidase (HRP)-coupled antirabbit-IgG (1:20,000) as a secondary antibody. GCS was detected by the HRP-activated chemiluminescence substrate using Lumiglo (Kirkkegaard, Gaithersburg, MD).

Northern blot analysis

Total RNA of hairpin HeLa cells was isolated using the RNAeasy kit according to the manufacturer's instructions. RNA (5–15 μg) was separated by 3% (w/v) agarose gel electrophoresis and transferred to Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Northern blot was performed as described by Sambrook et al. (2002). The blots were prehybridized with Church buffer (Sambrook and Russel, 2002) and hybridized with a ^{32}P-labeled cDNA probe (labeling was performed using Megaprime Labeling Kit, Amersham Bioscience) of either GCS, pSAP, or oligoadenylate synthase (OAS) at 50°C for 16 hours. The membranes were washed in 2× SSC twice for 20 minutes at ambient temperature and 0.1 × SSC/0.1% SDS at 68°C and analyzed with a Fuji Image Analyzer BAS1000 (Fuji Photo Film Co. Ltd., Tokyo, Japan). RNA oligonucleotides were used as markers.

Immunocytochemistry

Double immunofluorescence labeling of pSAP and the cytosolic protein β-adaptin was performed. HeLa cells grown on glass coverslips were washed with PBS+ (1× PBS, 0.1 M $CaCl_2$, 0.1 M $MgCl_2$), fixed in 4% (w/v) paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS+. Cells were incubated with primary antibodies: mouse monoclonal antirat β-adaptin antibody—1:1000 in 0.1% (v/v) Triton, 1% bovine serum albumin (BSA) in PBS+—for 1 hour at ambient temperature and washed three times with PBS+, and rabbit antihuman SAP-D antibody—1:500 in 0.1% (v/v) Triton, 1% BSA in PBS+. The mouse monoclonal antirat β-adaptin antibody was generously provided by T. Kirchhausen (Har-

vard Medical School, Boston, MA). The rabbit poly-clonal antihuman SAP-D antibody was made in K. Sand-hoff's laboratory. Cytosolic β-adaptin was visualized with Alexa 594-conjugated goat antimouse secondary antibody (Molecular Probes, Eugene, OR) (1:1000) in 0.1% Triton, 1% BSA in PBS+, applying the same conditions as described for the primary antibody. The SAP-D antibody was detected with Alexa 488-conjugated goat antirabbit secondary antibody. Finally, cells were washed in PBS and mounted on the microscope slide with antibleach mounting medium (25% phenylendiamin, 80% glycerol).

Microscopy and image analysis

Fluorescent microscopy was performed with a confocal laser scanning unit (LSM) coupled to a Zeiss Axiovert S100 (laser lines: argon 488 nm and HeNe 546 nm; filters for green fluorescence bandspan 505–550 nm, for red fluorescence bandspan 560 nm, line averaging 2×, 4×). Images were recorded with equal exposure times for specific antibodies. In each experiment, at least 10 coverslips were analyzed, and gene expression was quantified in both control and targeted cells by visual counting.

Metabolic labeling of glycosphingolipids with ^{14}C-serine pulse

Metabolic labeling and extraction of lipids were performed as described previously (Klein et al., 1994). Wild-type and pGCShp-cDNA3.1-transfected cells were cultured to confluency in 6-well plates. Medium was removed, and cells were washed twice with 1 ml serum-free MEM containing 1% penicillin/streptomycin per well. MEM (1 ml) containing 0.3% FBS and ^{14}C-serine (1 μCi/ml) was added to each well, and cells were incubated for 24 hours at 37°C/5% CO_2.

Chase

Pulse medium was removed, and cells were washed twice with 1 ml serum-free MEM containing 1% penicillin/streptomycin. MEM (2 ml) containing 0.6% FBS and unlabeled serine (10-fold excess of the ^{14}C-serine) was added, and cells were incubated for 120 hours at 37°C/5% CO_2.

After 120 hours of chase, the medium was removed, and cells were washed twice with 1 ml PBS, trypsinized, and transferred into 15-ml plastic tubes. After centrifugation for 10 minutes at 2000 rpm, supernatant was removed, and cells were frozen at −20°C for at least hours. Cells were resuspended in 600 μl water and sonicated for 30 seconds. Methanol (2 ml) was added, and the contents were transferred to a glass tube and sonicated. After the addition of 1 ml chloroform and sonication, probes were incubated for at least 2 hours at 37°C. To remove cell debris, probes were centrifugated at 4000 rpm for 15 min-

utes, and supernatants were transferred into new glass tubes. The solvent was evaporated.

Alkaline methanolysis

The dried lipid was sonicated for 5 minutes in 2.5 ml methanol, 25 μl 5 M NaOH. The samples were incubated for at least 2 hours at 37°C and neutralized with 7 μl glacial acetic acid, and the solvent was evaporated in a nitrogen stream.

Reverse phase chromatography

After the alkaline treatment, samples were dissolved in 2 ml ammonium acetate (150 mM in 50% methanol). Prior to thin layer chromatography (TLC), the samples were desalted by reverse phase chromatography. RP18 (40–63 μm) columns (1.5 ml) were prepared in Pasteur pipettes and equilibrated with a solution of chloroform/methanol/0.1 M potassium chloride (3/48/47, v/v/v). After loading of the samples, columns were washed with water, and the lipids were eluted with 1:1 chloroform/methanol.

Separation of cerebrosides

Dried lipids were resuspended in 1 ml chloroform/methanol/water (3/7/1, v/v/v) and loaded onto columns containing 1 ml equilibrated DEAE-sepharose, and the flow through recuperated. The column was washed with 4 ml chloroform/methanol/water (3/7/1, v/v/v), and the flowthrough recuperated.

Separation of gangliosides

Gangliosides were eluted with 6 × 1 ml chloroform/methanol/1 M ammonium acetate (3/7/1, v/v/v). Both ganglioside and cerebroside fractions were lyophilized and resolved in 100 μl 1:1 chloroform/methanol.

TLC

Lipid amounts containing equal radioactivity were spotted onto TLC plates (HPTLC, Merck, Darmstadt, Germany) and developed with a solvent chloroform/methanol/(0.22%) (w/v) $CaCl_2$ in water (60:35:8, v/v/v). The TLC samples were exposed to a PhosphoImager screen and analyzed with a Fuji Image Analyzer BAS1000 (Schepers et al., 1996).

RESULTS

Endogenously expressed dsRNAs efficiently and specifically suppress gene expression in cultured human cells

To assess the idea that endogenously expressed dsRNA molecules encompassing several hundred nucleotides

can be used to overcome the problems related to the dsRNA-dependent nonspecific IFN response in cultured mammalian cells, we engineered human cell lines expressing long RNA inverted repeats targeting the genes coding for GCS, GBA, and pSAP for RNAi. These 1600-nt-long RNA inverted repeats comprise 800 nt of a target cDNA in a consecutive sense and antisense orientation (Fig. 1).

In contrast to those plasmids expressing siRNAs hairpins (Brummelkamp and Agami, 2002; Yu et al., 2002) and those expressing large dsRNA (Paddison et al., 2002b), our constructs do not harbor a large spacer between the inverted fragments. Indeed, the loop formed by

the spacer is known to negatively influence the efficiency of the dsRNA to trigger RNAi. This decrease in efficiency seems to be related to the length of the spacer (Smith et al., 2000) and to its nucleotide composition (Brummelkamp and Agami, 2002). In this study, our two fragments are directly linked, ensuring the formation of a loop with a minimum of 6 nt, which also is palindromic. Further loops that differ in their composition and length were not tested.

We used two strategies to design our plasmids. To design the plasmid destined to specifically silence the GCS gene, we generated two DNA fragments of 800 nt from the first 800 bp of the cDNAs encoding for the GCS protein. The two DNA fragments differ by their 5'-sense restriction sites (BamHI and XhoI) originated from the forward primer. They share the restriction site (EcoRI) originated from the reverse primer. In a triple ligation, both fragments were inserted into the BamHI/XhoI site of pcDNA3.1, thereby generating an inverted repeat of 1600 bp (Fig. 1). For the plasmids destined to specifically silence the pSAP and GBA genes, we only generated one fragment carrying two restriction sites for BamHI (from the forward primer) and EcoRI (from the reverse primer). This fragment was inserted in a sense and an antisense orientation, into the BamHI (EcoRI) site of pcDNA3.1, also leading to the formation of a DNA inverted repeat of 1600 bp (Fig. 1).

Generation of stably transfected cell lines

To test the ability of our dsRNA-expressing plasmids to trigger dsRNA-induced RNA silencing or RNAi, we generated different human cell lines stably expressing the respective dsRNAs. The GCS, pSAP, and GBA hairpin dsRNA-expressing vectors, pGCShp-cDNA3.1, pSAPhp-cDNA3.1, and pGBAhp-cDNA3.1 (Fig. 2), were used to express constitutively active RNAi knockout phenotypes of GCS, pSAP, and GBA in differently cultured mammalian somatic cell lines, such as HeLa cells, human primary fibroblasts, kidney epithelial cells, myocytes, and mouse melanoma cells (MEB4). Following the transfection, stably transfected cell clones of the different cell types were selected for 30–40 days against G418 (300–1200 μg/ml) and subsequently analyzed by RT-PCR, Western blot, immunofluorescence, and glyco-sphingolipid composition. Because of the strong CMV promoter, controlling the expression all three constructs could generate complete RNAi knockout phenotypes in almost all selected stably transfected cell clones. Some selected cell clones show a knockdown of gene expression that might be due to low expression levels of the hairpin construct (data not shown). After selection for almost 6 months, no IFN response, PKR activation, or apoptosis was observed, and the cells were viable and healthy. As one example, levels of 2′,5′-oligoadenylate
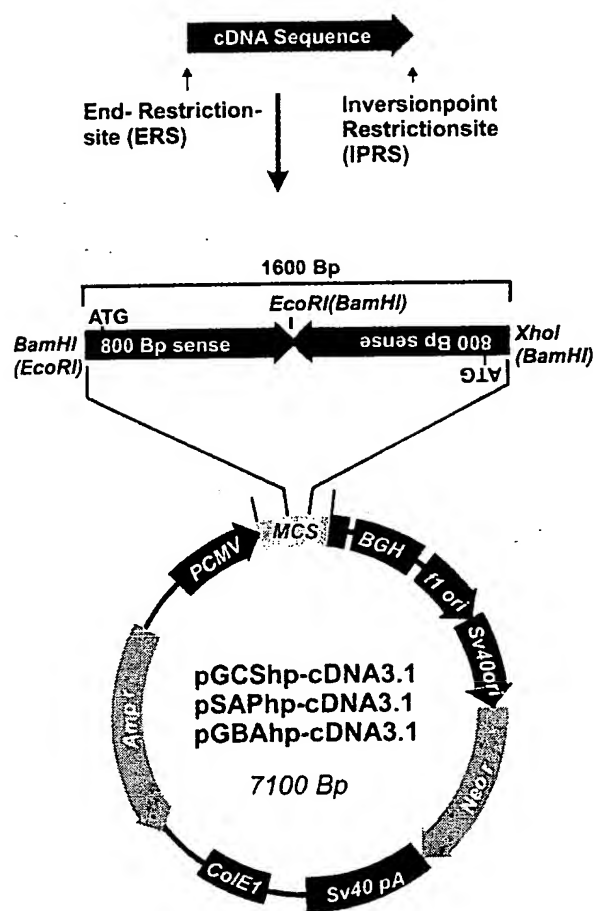

FIG. 1. Schematic view of the inverted repeat or hairpin vectors for endogenous production of dsRNA. The resulting stably transfected cell lines carry a plasmid that contains a stretch of DNA encoding for the first 800 nt of the target mRNA (GCS) in a sense and antisense orientation (inverted repeat). During transcription of the inverted repeat sequence, an RNA molecule is formed that is supposed to fold back into a hairpinlike structure by intramolecular hybridization generating dsRNA. This dsRNA will then be processed into siRNAs of 21–23 bp that induce degradation of the homologous endogenous GCS mRNA.

hairpin-
transgenic
HeLa cells
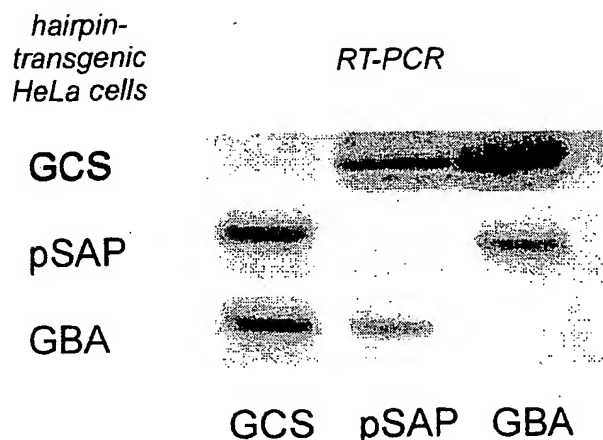
RT-PCR

GCS

pSAP

GBA

GCS   pSAP   GBA

FIG. 2. RT-PCR analysis of pGCShp-cDNA3.1, pSAPhp-cDNA3.1, and pGBAhp-cDNA3.1 stably transfected HeLa cells. As an example, we show the result of stably transfected hairpin HeLa cells. RT-PCR was carried out using primers comprising the sequence of the first 800 nt of the GCS, pSAP, and GBA mRNA. As controls, we performed other RT-PCRs to amplify the segments of the first 800 nt of the other cDNAs. As expected, RT-PCR of stably transfected cells did not show mRNA amplification, whereas the controls could be amplified.

synthase were analyzed in a Northern blot (see Fig. 7B). After 6 months of selection, cells were repeatedly frozen and thawed and cultured without G418 for several months. All hairpin-expressing cells kept the phenotype, with the exception of the melanoma cells, which lost the phenotype without selection.

RT-PCR of hairpin HeLa cells revealed no traces of the respective mRNAs

To prove the efficacy of GCS, pSAP, and GBA RNA silencing in HeLa cells, we isolated total RNA from the cells and performed RT-PCR analysis of the GCS, pSAP, and GBA mRNA using primers comprising the open reading frame (ORF) of the respective cDNAs. As controls, we amplified the first 800 bp of GCS, pSAP, and GBA. The amount of total RNA used in this analysis was normalized by the amount of 18S and 23S RNA (data not shown).

In RT-PCR experiments with GCS hairpin cells, no traces of GCS mRNA could be amplified, whereas the control amplifications of pSAP and GBA mRNA show no knockdown effect (Fig. 2). The same results could be obtained analyzing the pSAP and GBA cells using amplification of the other mRNAs as controls (Fig. 2). The results indicate that the long, endogenously produced dsRNA does indeed mediate a sequence-specific cleavage of GCS, pSAP, and GBA mRNA, leading to silencing of GCS, pSAP, and GBA genes in somatic cells.

Besides HeLa cells, gene silencing can be induced in other somatic cells

To verify the result of the RT-PCR, we determined the GCS protein content in immunoblots. Using a rabbit anti-GCS antibody (kindly provided by R. Pagano) as primary antibody and an HRP-linked antirabbit goat IgG antibody, cell lysates of GCS hairpin transfected HeLa cells, human primary fibroblasts, and melanoma cells (MEB4) were analyzed to detect the presence of the respective protein. As controls, we analyzed wild-type (wt) untransfected cells. In contrast to wt cells, no protein could be detected in the RNAi cells (Fig. 3). This result confirmed the data observed by RT-PCR and indicated that the absence of the respective protein is due to the sequence-specific, dsRNA-mediated degradation of the corresponding GCS mRNA. Because of the low abundance of GCS in HeLa cells, the signal in HeLa cells was very low. However, the result using HeLa cells was supported by RT-PCR (Fig. 2) and Northern blot analysis (see Fig. 7).

Immunocytochemical analysis and other visual assays show knockout phenotypes

We analyzed different somatic cell lines for their suppression of gene expression, which occurred on stable expression of the hairpin constructs pSAPhp-cDNA3.1 and pGCShp-cDNA3.1. As one assay, we tested the effects *in situ* by counting fluorescent cells in an immunocytochemical analysis of pSAPhp-HeLa cells vs. wt HeLa cells. As expected from the Western blot analysis for GCS, no pSAP expression could be detected in pSAPhp-cDNA3.1 HeLa cells, whereas the control protein β-adaptin was still present (Fig. 4). All cells on the coverslip showed the same phenotype, as they derived from the same selected cell clone. We did not observe any cell with positive pSAP staining, making FACS anal-
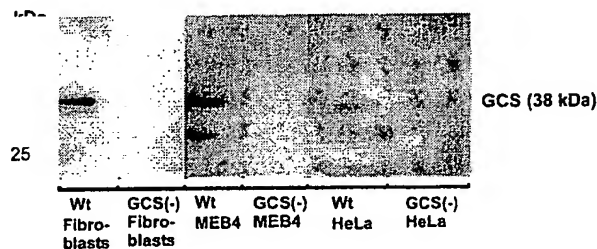
GCS (38 kDa)

25

Wt   GCS(-)   Wt   GCS(-)   Wt   GCS(-)
Fibro-   Fibro-   MEB4   MEB4   HeLa   HeLa
blasts   blasts

FIG. 3. Western blot analysis. To verify the results of the RT-PCR, we analyzed the RNAi on GCS protein activity in HeLa cells, human primary fibroblasts, and mouse melanoma (MEB4) cells. We performed Western blot analysis to detect GCS using a rabbit anti-GCS antibody. As a control, we used nontransfected HeLa cells, MEB4 cells, and human fibroblasts. Compared to nontransfected cells, no protein was detected in the RNAi cells.
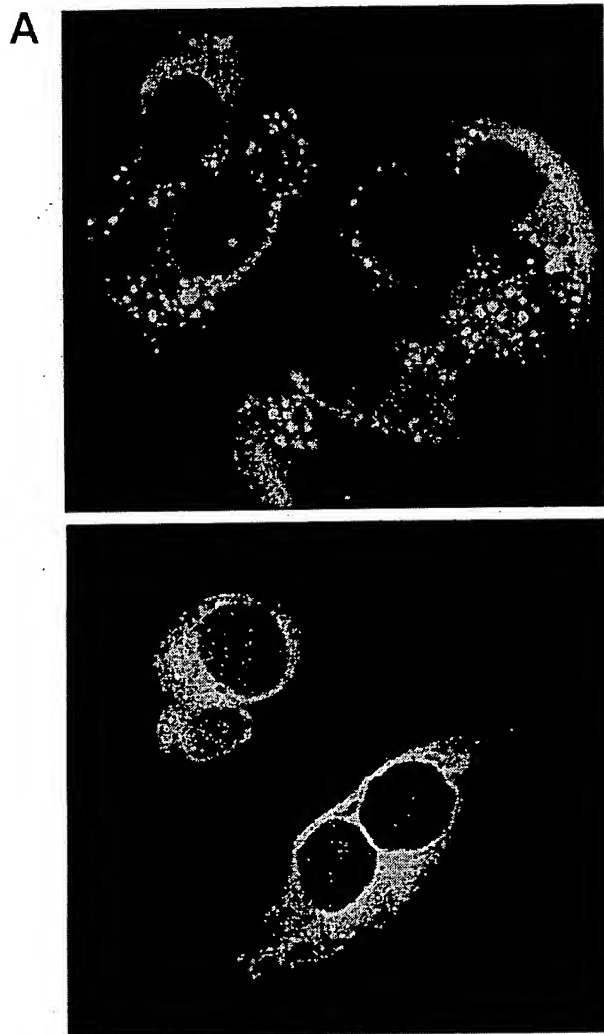
FIG. 4. Immunofluorescence of pSAPhp-cDNA3.1 HeLa cells. Stable suppression of gene expression. HeLa cells were transfected with 2 μg pSAPhp-cDNA3.1 vector and selected with increasing amounts of G418 (300 μg/ml–1.2 mg/ml) for 4 weeks. Clones were picked, expanded, and analyzed for pSAP protein levels. Micrographs show the merge of both images. (A) Wild-type HeLa cells. Immunofluorescence using antibodies against the processed lysosomal form of pSAP (anti-SAP-D antibody) and against β-adaptin, as a cytosolic control. (B) pSAPhp-cDNA3.1 HeLa cells. No fluorescence could be detected in the lysosomal structures.

the synthesis of melanin and pigmentation. Inhibition of this step completely blocks the synthesis of melanin and pigments. Sprong et al. (2001) also showed that other melanosome-specific proteins reached their final destination, suggesting that tyrosinase is transported within vesicles enriched in glucosylceramide (GlcCer), which is generated by the addition of a glucosyl residue on ceramide catalyzed by GCS. As a result of production of pigment, cell pellets from wt melanoma cells are almost black. Transfection of these cells with pGCShp-cDNA3.1 and selection of a pGCShp-cDNA3.1 melanoma cell line revealed discoloring of the cells, assuming that the knockout of GCS was successful (Fig. 5). Western blot analysis on the transfected cell line (Fig. 3) and metabolic labeling of the lipids (data not shown) supported the results published by Sprong et al. (2001).

Metabolic labeling of lipids: a sensitive method to measure potency of knockout phenotypes

As mentioned, GCS catalyzes the transfer of glucose from UDP-glucose to ceramide, leading to the formation of GlcCer (Fig. 6) (Diallo et al., 2003; Kolter, 1999; Schuette et al., 2001). This reaction occurs on the cytosolic side of the Golgi membrane and is the first step in the genesis of the most important classes of glycosphingolipids, including the gangliosides (Kolter, 1999). Silencing of GCS expression would have an inevitable consequence on the natural glycosphingolipid composition in the plasma membrane of stably transfected RNAi
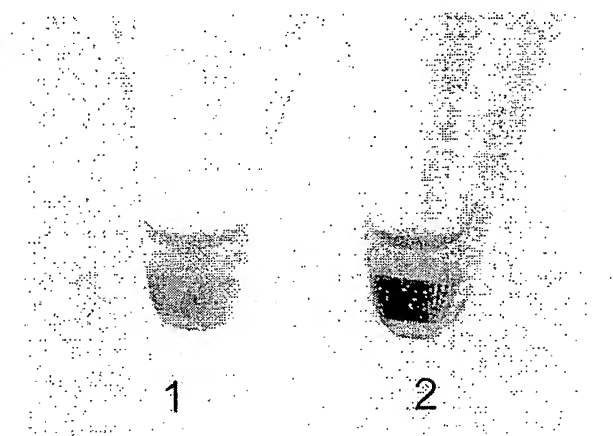

FIG. 5. Knockout with RNA hairpins. Stable expression of pGCShp-cDNA3.1 in murine melanoma cells disrupts the synthesis of GCS. A deficiency of glucosylceramide blocks melanin processing by tyrosinase. Tyrosinase is probably retained within the Golgi apparatus and is not transported to the melanosomes, where it is supposed to process melanin. Therefore, melanoma cells lose their pigmentation and turn from dark brown to almost white. (1) Pellets of pGCShp-cDNA3.1 MEB4 cells and (2) wt MEB4.
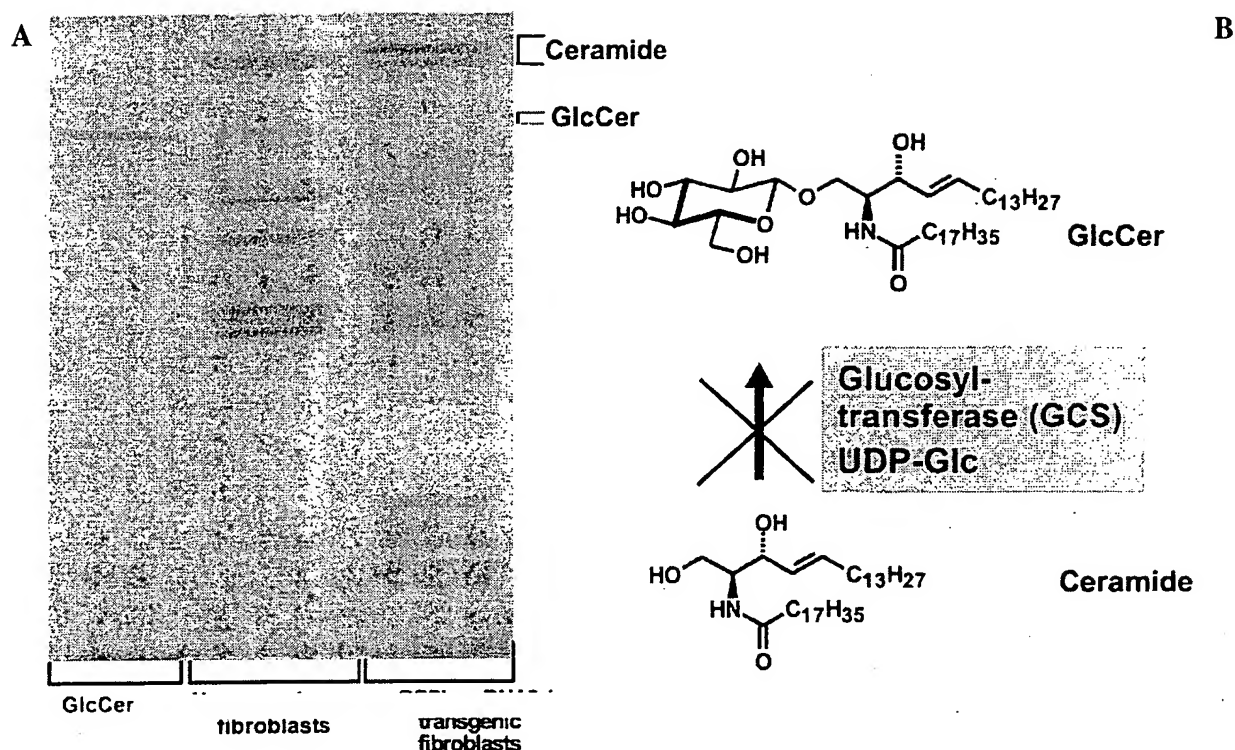
ysis unnecessary. Another visual assay to survey GCS protein expression is based on the pigmentation of MEB4 cells (Fig. 5). Van Meer's group (Sprong et al., 2001) reported that in a GCS knockout mouse melanoma cell line, tyrosinase was mislocated or retained within the Golgi apparatus, whereas in wt melanocytes or melanoma cell lines, it was usually transported within specialized vesicles to the melanosomes. There, it converted tyrosine to L-DOPA, which is the first rate-limiting step in

FIG. 6. Metabolic labeling of glycosphingolipids in human primary fibroblast. To study the lipid distribution in pGCShp-cDNA3.1 cells compared with wt cells, we analyzed the phenotype in human primary fibroblasts cells. The glycolipids were metabolically labeled with ^{14}C-serine, and the lipids were extracted. Separation of the lipids was performed in a TLC on silica plates. (A) Autoradiograph of the radioactively labeled lipids. (B) A schematic view of the reaction of the UDP-glucose transfer onto ceramide to yield GlcCer, which is catalyzed by the GCS. As expected, GlcCer synthesis is blocked, and the ceramide (Cer) levels seem to be enhanced in GCS-deficient pGCShp fibroblasts. As standards we used ^{14}C*-labeled C_{18}-GlcCer (kindly provided by G. van Meer, Utrecht) and C_{18}-ceramide (N-stearoyl-D-sphingosine) (Sigma, Taufkirchen, Germany). Comparison with the nonlabeled ceramide marker was made by visualization of the marker with cupric sulfate in aqueous phosphoric acid (van Echten-Deckert, 2000).

cells. GCS is of particular interest for a number of reasons. Sphingolipids, particularly glycosphingolipids with GlcCer as a precursor, have a propensity to cluster in an environment of other lipids, including cholesterol, forming lateral microdomains on the outside of the plasma membrane that are distinct from those of bulk membrane (Brown and London, 2000; Hakomori, 1998). Several cellular membranes contain high glycosphingolipid levels, for example, myelin and apical epithelial membranes, where they constitute up to 20–35 mol% of total lipid. They are thought to mediate insulation and protection of the membranes.

Therefore, we measured the impact of the pGCShp-cDNA3.1 expression on lipid composition. We extracted the glycosphingolipids from stably transfected pGCShp-cDNA3.1 and wt human primary fibroblasts and determined their composition by TLC on silica plates (Fig. 6). As expected, we found that the composition of glyco-sphingolipids in those stably transfected cells is dramatically changed compared with wt cells. GlcCer was com-

pletely depleted, whereas the ceramide levels were increased due to the block in the metabolic pathway (Fig. 6B). The inhibition of GlcCer synthesis in those cells seems to influence the *de novo* synthesis of a completely different set of lipids, which still have not been identified in detail.

Endogenously expressed dsRNA will be processed into siRNAs

To determine if this type of RNA silencing can be connected to RNAi and if the endogenously expressed hairpin dsRNAs will be processed into 21–23-mer, a Northern blot analysis was performed (Fig. 7). RNA from both transgenic cell lines, pGCShp-cDNA3.1 and pSAPhp-cDNA3.1, transgenic HeLa cells, and wt HeLa cells was isolated, transferred onto a nylon membrane, and probed with ^{32}P-labeled human cDNAs of GCS and pSAP (Fig. 7A). The Northern blot showed that compared to wild type cells transfected with pGCShp-cDNA3.1 and
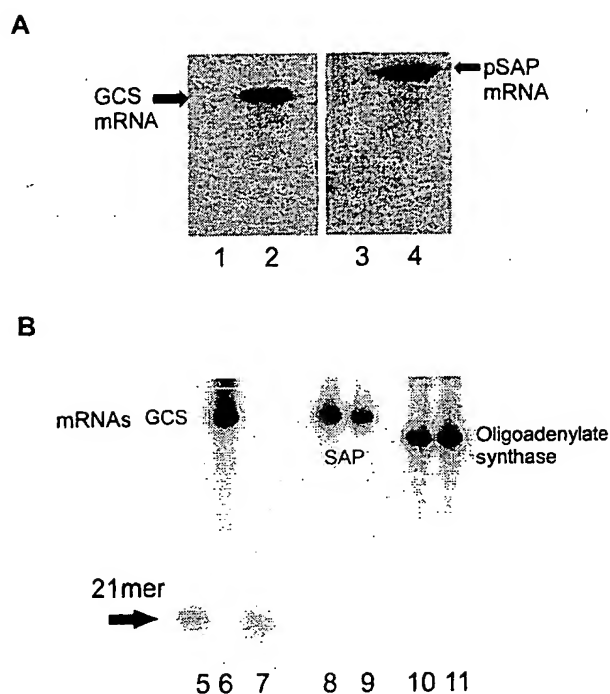
A

B

FIG. 7. Northern blot analysis in pGCShp-cDNA3.1 and pSAPhp-cDNA3.1 HeLa cells. (A) Northern blots of RNA extracted from pGCShp-cDNA3.1 (lane 1) and pSAPhp-cDNA3.1 (lane 3) stably transfected HeLa cells and wt HeLa cells (lanes 2 and 4) show the deficiency of the corresponding mRNA in hairpin-expressing cells but not in control cells. Blots were probed with the ^{32}P-labeled cDNA of GCS (lanes 1 and 2) or pSAP (lanes 3 and 4). (B) Northern blots of RNA extracted from wt HeLa cells (lane 6) and pGCShp-cDNA3.1 (lane 7) show the deficiency of the corresponding mRNA and production of a 21-mer GCS-RNA species in pGCShp-cDNA3.1 cells (lane 7) but not in wt HeLa cells (lane 6). As a control, we probed wt HeLa cells (lanes 8 and 10) and pGCShp-cDNA3.1 cells (lanes 9 and 11) with a ^{32}P-labeled cDNA of pSAP (lanes 8 and 9) or a ^{32}P-labeled cDNA of 2′,5′-oligoadenylate synthase (lanes 10 and 11). As a marker for the 21-mer species, we used a 21-mer GCS sequence-based RNA oligo (lane 5).

pSAPhp-cDNA3.1 did not produce mature GCS mRNA or pSAP mRNA. The 1600-nt inverted repeat could not be detected with either probe, suggesting rapid cleavage to siRNA.

In a second Northern blot analysis, RNA from pGC-Shp-cDNA3 HeLa cells and from wt HeLa cells was probed with ^{32}P-labeled cDNAs of GCS, pSAP, and 2′,5′-oligoadenylate synthase. As a marker, we used a 21-mer GCS RNA oligonucleotide, which was enzymatically synthesized using a T7-based system (K. Schmitz, unpublished data). Cells transfected with pGCShp-cDNA3.1 produce small RNA fragments, which are similar in size to the 21-nt marker (Fig. 7B). Again, using this type of analysis, no mature GCS mRNA was detected, whereas it was evident in wt cells. These results

indicate that the hairpin dsRNA transcript was generated and cleaved in the cell to produce functional small RNAs. In both, wt and pGCShp-cDNA3 HeLa cells, the control mRNAs, pSAP and 2′,5′-oligoadenylate synthase were expressed at normal levels, showing specificity and no obvious activation of an IFN response.

DISCUSSION

Since its discovery, RNAi has been developed as an important tool for reverse genetics, being routinely used to knock down gene function in such diverse organisms as *C. elegans*, *Drosophila*, *Neurospora crassa*, and plants. The existence of a very similar RNA silencing mechanism in mammalian cells, subsequently termed RNAi, suggests that this procedure could also be used as a powerful tool for functional genomics in mammals (Arenz and Schepers, 2003; Schepers and Kolter, 2001). The first indications that mammals can also induce dsRNA-dependent RNA silencing came from the observation that injection of dsRNAs into early mouse embryos, mouse embryonic cells, and even whole mice induced sequence-specific silencing (Paddison et al., 2002b; Svoboda et al., 2000, 2001; Wianny and Zernicka-Goetz, 2000).

Finally, the work of Tuschl's group (Elbashir et al., 2001) has revolutionized RNAi in somatic mammalian cells. Following on previous studies that identified siRNAs as mediators of RNAi, they made the remarkable finding that transfection of synthetic 21-nt siRNA duplexes into mammalian cells effectively silences endogenous genes. Although these siRNAs are probably too short to trigger the IFN response, they are able to direct sequence-specific cleavage of homologous mRNAs (Elbashir et al., 2001; Bridge et al., 2003). However, this application in cell culture, as well as the use of large dsRNAs in mouse embryos (Wianny and Zernicka-Goetz, 2000), is transient, which severely restricts these applications. To overcome this limitation, several groups generated mammalian expression vectors that direct the synthesis of endogenous dsRNA or siRNA transcripts. These plasmids were used either for *in vitro* transcription of siRNA hairpins, taking advantage of a T7-polymerase recognition sequence (Donze and Picard, 2002), or for direct expression of siRNA hairpins driven by the strong pol III promoter.

The first system, which combines both approaches of persistent RNAi and siRNA application, was developed by Brummelkamp and Agami (2002), followed by several other groups (Lee et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002a; Paul et al., 2002; Sui et al., 2002). The vector systems they used code for short hairpins simulating siRNAs, which mediate permanent suppression of gene expression and allow analysis of loss-of-function phenotypes that develop over longer periods

of time. In none of these attempts, however, could a 100% knockdown of the activity of the targeted gene be achieved. Likewise, Tuschl et al. (1999) realized a knockdown of only 80%–90% using their synthetic siRNAs. The concerns of remaining activity might be explained by poor transfection efficiency or insufficient internalization or both, which can be the limiting factors. Although short dsRNAs do not mediate RNAi efficiently (Parrish et al., 2000; Tuschl et al., 1999; Yang et al., 2000), regions of homology between the dsRNA and the target gene as short as 23 nt can mediate posttranscriptional gene silencing in tobacco, when the homology is contained within a longer dsRNA. The fact that purified siRNAs cleaved from long dsRNAs (Nykanen et al., 2001) can efficiently mediate RNAi *in vitro* suggests that long dsRNAs are more effective because they are more efficiently processed into siRNAs, possibly because of a highly cooperative binding or cleavage by Dicer (Zamore, 2001).

In this report, we show that despite some concerns, which are discussed within the field of RNAi, long endogenously expressed hairpin dsRNAs are capable of inducing an RNAi-like RNA silencing phenotype in mammalian somatic cells, including human primary fibroblasts. We generated several stably transfected somatic cell lines, for example, HeLa cells, primary fibroblasts, melanoma cells, and others, that produce hairpin dsRNAs displaying about 800 nt in each sense and antisense direction. During transcription of the inverted repeat sequences, an RNA molecule is formed that is supposed to fold back into a hairpin-like structure by intramolecular hybridization. The resulting RNA should be effectively double stranded. We consider that these dsRNAs will be processed by the mammalian Dicer into the 21–23-nt siRNAs that will be able to induce specific silencing of the corresponding gene in the same way as it occurs in *C. elegans* and *Drosophila*.

An important aspect of this method is that the siRNAs processed from the dsRNAs by Dicer are available in their natural form and, therefore, could exhibit stronger potency in RNAi than the synthetic ones used by Tuschl's group or those expressed as siRNA hairpins by Agami's group.

After transfection of the constructs and an extensive period of selection with G418 (geneticin), we gained cells with permanent null phenotypes for various targeted enzymes of the glycosphingolipid metabolism. The reason why such cells do not undergo programmed cell death is unknown. Apparently, endogenously produced dsRNA is not active or is less active in inducing the IFN response than is exogenous dsRNA. Despite some recent reports (Bridge et al., 2003; Sledz et al., 2003) that demonstrate that short hairpin RNAs (shRNAs) could induce the IFN response, we did not observe any induction of the IFN response. Analysis of 2′,5′-oligoadenylate syn-

thase levels by Northern blot showed equal levels of mRNA. A possible explanation for this observation is that the endogenous dsRNA is rapidly processed to smaller fragments that are eventually processed into active siRNAs, avoiding an accumulation of longer dsRNA in the cytosol. One could argue that we have used tumor cells, such as HeLa or melanoma cells, that often do not induce an IFN response. However, we could not detect any apoptosis in the primary fibroblasts. In addition, the IFN response could be triggered after interaction of exogenous dsRNA longer than 30 nt with proteins localized on the outside of the cell membrane and could induce a nonspecific response, whereas this interaction does not occur if dsRNAs are endogenously expressed. It has been suggested that only those cells can escape selection that produce long hpRNA in levels too low for IFN response induction but sufficient for interference with endogenous mRNAs. There have been reports on the antagonistic effect of RNAi and adenosine deamination of dsRNA by adenosine deaminases that act on dsRNA (ADARs). ADARs directly target dsRNA and inhibit the processing of siRNAs (Scadden and Smith, 2001). One could speculate that in our cell clones that survived the extensive selection procedure, the levels of siRNAs may be reduced by deamination of the overexpressed dsRNA.

Although the cloning efficacy for long inverted repeats of cDNA is very low and selection of the cells is very time consuming, this technique is valuable for long-term studies, which demand downregulation rates of gene expression to a higher extent than is achievable by expression of siRNAs or siRNA transgenes. One of the limiting factors of all methods to induce RNAi in mammals and other species, however, is the turnover of the target protein. There are proteins displaying short vs. long half-lives and low vs. high abundance within a cell or organism. Thus, knocking down the biosynthesis of a highly abundant protein, such as actin or overexpressed proteins, would require a higher dosage of siRNA or dsRNA, whereas in the case of a protein with a long half-life, one has to wait longer to see a knockdown effect on the protein level. Some proteins are stored within the cell and have turnover rates of longer than weeks, which makes it difficult to analyze them using this technique. A good example of a protein with a long half-life is clathrin. It is stored at the plasma membrane in large and persistent pools and is hard to deplete (personal observation; Motley et al., 2003).

As has been shown for *C. elegans*, RNAi is a very powerful tool in high-throughput reverse genetic studies (Ashrafi et al., 2003; Gonczy et al., 2003; Kamath et al., 2003), and there are similar studies in progress for mammalian cells. Our system to silence gene expression is probably too laborious to perform genomewide screens, but it has great potential for application in whole mammalian organisms, including the challenge of generating

transgenic mouse models to study gene function *in vivo*. This will assist in elucidating gene functions in numerous cell types and tissues, including primary cells.

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